

## DESCRIPTION

METHOD OF SCREENING FOR COMPOUNDS THAT INHIBIT THE ENZYMATIC ACTIVITY OF GWT1 GENE PRODUCT

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Technical Field

The present invention relates to methods of screening for antifungal agents having the activity of inhibiting GPI synthase, which is involved in the synthesis of fungal cell walls.

10 Background Art

The present inventors noticed that adhesion to host cells is important for fungi to exert their pathogenicity, and that adhesion factors involved in fungal cell adhesion are transported to the surface layers of cell walls after glycosylphosphatidylinositol (GPI) anchors on the cell membrane (Non-Patent Document 1: Hamada K *et al.*, Mol. Gen. Genet., 258: 53-59, 1998).

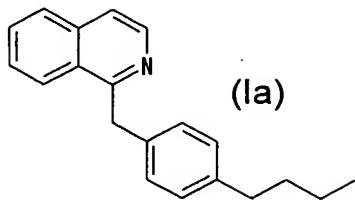
15 Accordingly, the present inventors considered that novel antifungal agents that inhibit the synthesis of fungal cell walls and also inhibit the adhesion of fungal cells to host cells could be generated by inhibiting the process of transporting proteins anchored with GPI (GPI-anchored proteins) to cell walls. Thus, the present inventors started study.

20 Disclosure of the Invention

An objective of the present invention is to develop antifungal agents for preventing pathogenic fungi from exerting pathogenicity, by inhibiting the synthesis of fungal cell walls, as well as by inhibiting fungal cell adhesion to host cells, by inhibition of the transport of GPI-anchored proteins to fungal cell walls.

25 In WO 02/04626, the present inventors found the following proteins involved in the process of transporting GPI-anchored proteins to cell walls: the proteins of *Saccharomyces cerevisiae* encoded by DNAs comprising the nucleotide sequence of SEQ ID NO: 1; the proteins of *Candida albicans* encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 3 and 5; the proteins of *Schizosaccharomyces pombe* encoded by DNAs comprising the nucleotide sequence of SEQ ID NO: 7; the proteins of *Aspergillus fumigatus* encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 9 and 11; and the proteins of *Cryptococcus neoformans* encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 12 and 13. These nucleotide sequences were called GWT1 genes. In addition, the inventors found that GWT1 gene-deficient fungi can not synthesize cell walls. Furthermore, the inventors found 30 that the compound represented by formula (Ia) binds to the above-described proteins to inhibit the transport of GPI-anchored proteins to cell walls, thus inhibiting the synthesis of fungal cell 35

walls.



The inventors then found that the GWT1 gene product (hereinafter referred to as "GWT1 protein") has the activity of synthesizing GlcN-(acyl)PI by transferring an acyl group to 5 GlcN-PI in the GPI biosynthesis pathway (Fig. 1; Kinoshita and Inoue, *Curr Opin Chem Biol* 2000 Dec;4(6): 632-8; Ferguson *et al.*, *Biochim Biophys Acta* 1999 Oct 8; 1455 (2-3): 327-40). The inventors conceived that compounds inhibiting the synthesis of fungal cell walls could be found by screening for compounds that inhibit this activity, and thus completed the present invention.

10 Specifically, the present invention provides [1] to [4] as described below.

[1] A method of screening for a compound having an antifungal activity, wherein the method comprises the steps of:

- (1) contacting a test sample with an overexpressed protein encoded by the GWT1 gene;
- (2) detecting GlcN-(acyl)PI; and
- (3) selecting the test sample that decreases GlcN-(acyl)PI.

The "GWT1" gene refers to a gene involved in the synthesis of fungal cell walls, which was disclosed in WO 02/04626. The term "overexpressed" does not refer to expression of native genes, but to the expression of exogenously introduced genes.

"GlcN-(acyl)PI" refers to glucosaminyl-acylphosphatidylinositol in which an acyl group is linked with the inositol of glucosaminyl-phosphatidylinositol (GlcN-PI) in the GPI biosynthesis pathway (Fig. 1; Kinoshita and Inoue, *Curr Opin Chem Biol* 2000 Dec; 4(6):632-8; Ferguson *et al.*, *Biochim Biophys Acta* 1999 Oct 8; 1455(2-3):327-40).

[2] The method of [1], wherein the GWT1 gene is any one of the following:

- (a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 25 10, or 14;
- (b) a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 12, or 13;
- (c) a DNA hybridizing to the DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 12, or 13 under stringent conditions; and
- (d) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 30 10, or 14, wherein one or more amino acids have been added, deleted, substituted, and/or inserted.

The term "stringent conditions" means, for example, hybridization in 4x SSC at 65°C

followed by washing with 0.1x SSC at 65°C for one hour. Alternatively, stringent conditions refer to hybridization in 4x SSC with 50% formamide at 42°C. Other acceptable conditions may be hybridization in PerfectHyb™ solution (TOYOBO) at 65°C for 2.5 hours, followed by washing with (1) 2x SSC, 0.05% SDS at 25°C for five minutes; (2) 2x SSC, 0.05% SDS at 25°C for 15 minutes; and (3) 0.1x SSC, 0.1% SDS at 50°C for 20 minutes.

The “protein comprising an amino acid sequence in which one or more amino acids have been added, deleted, substituted, and/or inserted” can be prepared by methods known to those skilled in the art, for example, by site-directed mutagenesis (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 10 Laboratory, Cold Spring Harbor, NY). Such mutations can also occur naturally. There is no limitation on the number of amino acids to be mutated, as long as the resulting protein retains the activity of transferring an acyl group to GlcN-PI. The number of amino acids to be mutated is typically 30 or less, preferably ten or less, and more preferably three or less. There is no limitation on the position of the mutated amino acids, as long as the protein retains the activity 15 described above.

The proteins and protein mutants prepared using the above-described hybridization techniques normally have high homology (for example, 60% or higher, 70% or higher, 80% or higher, 90% or higher, or 95% or higher homology) to proteins consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, or 14 at the amino acid level. The amino acid sequence 20 homology can be determined using a BLASTx program (at the amino acid level; Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990). This program is based on the BLAST algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). When the amino acid sequences are analyzed using BLASTX, parameters of, for example, score= 50 and wordlength= 3 are used. Alternatively, when using 25 the Gapped BLAST program, the amino acid sequences can be analyzed by the method described by Altschul *et al.* (Nucleic. Acids. Res. 25:3389-3402, 1997). When the BLAST and Gapped BLAST programs are used, the default parameter values for each program are used. Specific procedures for these analyses are known in the art (<http://www.ncbi.nlm.nih.gov>).

[3] The method of claim 1 or 2, wherein the step of detecting the acylated GPI is 30 thin-layer chromatography.

[4] The method of any one of [1] to [3], wherein the method further comprises a step 4, of determining whether the selected test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall, whether the test sample inhibits the expression of a 35 GPI-anchored protein on a fungal cell surface, or whether the test sample inhibits the proliferation of a fungi.

Methods for preparing GWT1 protein [1], and methods for determining transacylation activity[2] of the present invention are disclosed below.

### 1. Methods for preparing GWT1 protein

GWT1 protein is prepared from a fungal membrane fraction, preferably that of *S. cerevisiae*, *C. albicans*, *S. pombe*, *A. fumigatus*, or *C. neoformans*, and more preferably *S. cerevisiae*. The transacylation activity may be determined by using the prepared membrane fraction directly or after purification. The transacylation activity can be readily measured by introducing a DNA of the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 12, or 13 into 10 fungal cells to overexpress the GWT1 protein. This procedure can be specifically described using *S. cerevisiae*, as follows:

#### (1) Introduction of the GWT1 gene

The GWT1 gene can be prepared by carrying out PCR using fungal DNAs as templates, 15 and primers designed based on a nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 12, or 13.

The GWT1 expression plasmid is prepared by inserting an appropriate promotor and terminator, such as a GAPDH promoter and a GAPDH terminator derived from pKT10 (Tanaka *et al.*, Mol. Cell Biol., 10: 4303-4313, 1990), into the multi-cloning site of an expression vector that functions in *S. cerevisiae*, such as YEpl352, and inserting the GWT1 gene into the expression 20 vector. *S. cerevisiae* cells of, for example, G2-10 strain, are incubated while shaking in an appropriate medium such as yeast extract-polypeptone-dextrose (YPD) medium at an appropriate temperature, for example, at 30°C. The fungal cells are harvested at the late logarithmic growth phase. After washing, GWT1 expression plasmids are introduced into *S. cerevisiae* cells, for example, by the lithium acetate method. The lithium acetate method is described in the Users 25 Manual attached to YEAST MAKER™ Yeast Transformation System (Clontech). GWT1-overexpressing strain and empty vector-introduced strain can be obtained by culturing the cells in SD(ura-) medium at 30°C for two days.

Fungal strains to which the GWT1 gene is introduced are preferably deficient strains lacking their native GWT1 gene. *S. cerevisiae* GWT1 gene-deficient cells can be obtained by a 30 method described below.

PCR amplification is carried out using a marker gene, preferably *S. pombe* his5 gene, as a template, and primers designed to obtain PCR products which comprise 30 bp, or more preferably 40 bp or more of the GWT1 gene sequence (for example, the sequence of SEQ ID NO: 1) to be deleted. The resulting PCR products are purified, and then introduced into fungal 35 cells. Deficient strains can be obtained by screening appropriate to the marker gene, for example, by culturing the cells in his- medium when the marker is his5.

Expression vectors and gene introduction methods for fungus other than *S. cerevisiae* are described in: Igarashi *et al.*, Nature 353: 80-83, 1991, for *S. pombe* expression vector pcL and such, and methods for introducing the vectors; Pla J *et al.*, Yeast, 12: 1677-1702, 1996, for *C. albicans* expression vector pRM10 and such, and methods for introducing these vectors; Punt PJ 5 *et al.*, GENE, 56: 117-124, 1987, for *A. fumigatus* expression vector pAN7-1 and such, and methods for introducing these vectors; and Monden P *et al.*, FEMS Microbiol. Lett., 187: 41-45, 2000, for *C. neoformans* expression vector pPM8 and such, and methods for introducing these vectors.

Methods for preparing deficient strains of *C. albicans* are described in Fonzi WA *et al.*,  
10 Genetics 134: 717-728, 1993.

#### (2) Methods for preparing the membrane fraction

*S. cerevisiae* cells to which the GWT1 gene are introduced are cultured while shaking in an appropriate medium, such as SD(ura-) liquid medium, at an appropriate temperature, for 15 example 24°C. The fungal cells are harvested in the middle logarithmic growth phase. After being washed with TM buffer (50 mM Tris-HCl (pH 7.5) and 2 mM MgCl<sub>2</sub>), the fungal cells are suspended in an adequate amount (for example, 2 ml) of TM buffer + protease inhibitor (Complete<sup>TM</sup>; Roche). An adequate amount (for example, 1.5 ml) of glass beads is added to the suspension. The samples are vortexed and placed on ice, and these procedures are repeated (for 20 example, ten cycles of vortexing for 30 seconds and placing on ice for 30 seconds) to disrupt fungal cells.

The samples are centrifuged, for example, at 1000 g for five minutes, to precipitate glass beads and fungal cells which are not disrupted. The resulting supernatant is transferred to another tube, and then centrifuged, to precipitate the membrane fraction comprising organelles 25 (total membrane fraction), for example at 13 000 g for 20 minutes. If required, the precipitate is further suspended in 1 ml of an appropriate assay buffer, and centrifuged, for example, at 1000 g for one minute to remove those components which are not suspended. The supernatant is then centrifuged, for example, at 13 000 g for 20 minutes, and the resulting precipitate is resuspended in an appropriate assay buffer to obtain a membrane fraction.

30 Membrane fractions from fungal cells other than *S. cerevisiae* can be prepared by the methods as described in: Yoko-o *et al.*, Eur. J. Biochem. 257: 630-637, 1998, for *S. pombe*; Sentandreu M *et al.*, J. Bacteriol., 180: 282-289, 1998, for *C. albicans*; Mouyna I *et al.*, J. Biol. Chem., 275: 14882-14889, 2000, for *A. fumigatus*; and Thompson JR *et al.*, J. Bacteriol., 181: 444-453, 1999, for *C. neoformans*.

35 Alternatively, GWT1 protein can be prepared by expression in cells other than fungal cells, such as mammalian cells, insect cells, and *E. coli* cells.

When mammalian cells are used, a membrane fraction can be prepared by inserting GWT1 into, for example, an overexpression vector comprising CMV promotor; introducing the vector into mammalian cells; and then carrying out the method described in Petaja-Repo *et al.*, J. Biol. Chem., 276: 4416-23, 2001.

When insect cells are used, a membrane fraction can be prepared by preparing GWT1-expressing insect cells (such as Sf9 cells) using a baculovirus expression kit, for example, BAC-TO-BAC Baculovirus Expression system (Invitrogen); and then using the cells to carry out the method described in Okamoto *et al.*, J. Biol. Chem., 276: 742-751, 2001.

When *E. coli* is used, GWT1 protein can be prepared by inserting GWT1 into an *E. coli* expression vector, for example, pGEX (Pfizer); and then introducing the vector into *E. coli* cells such as BL21.

## 2. Methods for determining transacylation activity

The transacylation reaction to GPI can be detected by the method described in Costello and Orlean, J. Biol. Chem. (1992) 267: 8599-8603, or the method described in Franzot and Doering, Biochem. J. (1999) 340: 25-32. Examples of specific methods are illustrated below, however, the experimental conditions below are preferably optimized according to the GWT1 gene products to be used, as follows:

The GWT1 gene product prepared in Section 1, above, preferably a membrane fraction comprising a GWT1 gene product, is added along with test compounds to a buffer comprising: appropriate metal ions (Mg, Mn); ATP; and Coenzyme A; and preferably inhibitors that prevent the consumption of UDP-GlcNAc in other reactions, such as nikkomycin Z as an inhibitor of chitin synthesis, and tunicamycin as an inhibitor of the synthesis of asparagine-linked sugar chain. The mixture is incubated at an appropriate temperature for an appropriate period (for example, at 24°C for 15 minutes).

Then, a GlcN-(acyl)PI precursor (for example, UDP-GlcNAc or Acyl-Coenzyme A, and preferably UDP-[<sup>14</sup>C]GlcNAc) labeled with an appropriate label, preferably with an isotope, is added to the mixture. The resulting mixture is further incubated for an appropriate period (for example, for one hour at 24°C). A 1:2 mixture of chloroform:methanol is added to the mixture, and stirred to stop the reaction. Lipids are then extracted from the mixture. The extracted reaction products are dissolved in an appropriate solvent, preferably in butanol, and then subjected to HPLC, thin-layer chromatography (TLC), or such, and preferably TLC, to isolate GlcN-(acyl)PI generated in the reaction. A developing solvent for TLC can be selected appropriately, and may be, for example, CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (65:25:4), CHCl<sub>3</sub>/CH<sub>3</sub>OH/1 M NH<sub>4</sub>OH (10:10:3), or CHCl<sub>3</sub>/pyridine/HCOOH (35:30:7), and preferably HCl<sub>3</sub>/CH<sub>3</sub>OH/1 M NH<sub>4</sub>OH (10:10:3). The isolated GlcN-(acyl)PI is quantified by a method that accords with the

label used. When labeled with an isotope, the isolated GlcN-(acyl)PI is quantified based on its radioactivity.

When a reduced amount of GlcN-(acyl)PI is produced in the presence of a test compound, the test compound is determined to comprise the activity of inhibiting transacylation by GWT1 proteins.

A test sample found to comprise the activity of inhibiting transacylation as described above, is preferably further tested to determine whether it inhibits the process of transporting GPI-anchored proteins to fungal cell walls, whether it inhibits the expression of GPI-anchored proteins on fungal cell surfaces, or whether it inhibits fungal growth. If the test results show that the test sample inhibits the process of transporting GPI-anchored proteins to fungal cell walls, inhibits the expression of GPI-anchored proteins on fungal cell surfaces, or inhibits fungal growth, then the sample is a promising candidate for an antifungal agent.

Methods that (1) use reporter enzymes; (2) use antibodies that react to glycoproteins on the surface layers of fungal cell walls; (3) test fungal cells for adhesiveness to animal cells; or (4) observe fungal cells under a light microscope or electron microscope can be used to test whether a test sample inhibits the process of transporting GPI-anchored proteins to fungal cell walls or inhibits the expression of GPI-anchored proteins on fungal cell surfaces.

Methods (1) to (4) are enclosed in WO 02/04626, and specifically illustrated in the Examples. By using the methods of (1) to (4), preferably in combination, a test sample can be determined to inhibit the process of transporting GPI-anchored proteins to fungal cell walls or to inhibit the expression of GPI-anchored proteins on fungal cell surfaces. Further, a test sample can be determined to effect the process of transporting GPI-anchored proteins to cell walls, when the inhibition by the test sample is impaired or disappears when a protein encoded by a DNA of the present invention is overexpressed in fungal cells.

Conventional methods for measuring antifungal activity can also be used to determine whether a test sample inhibits fungal growth (National Committee for Clinical Laboratory Standards. 1992. Reference method for broth dilution antifungal susceptibility testing for yeasts. Proposed standard M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.).

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#### Brief Description of the Drawings

Fig. 1 shows the GPI biosynthesis pathway.

Fig. 2 is a photograph showing the results of determining GPI acylation in membrane fractions prepared from wild-type strain (WT), GWT1 gene-disrupted  $\Delta$ gwt1 strain ( $\Delta$ ), and GWT1 gene-introduced  $\Delta$ gwt1 strain ( $\Delta/G$ ).

Fig. 3 is a photograph showing the results of determining the GPI acylation inhibitory

activities of 1-(4-butylbenzyl)isoquinoline and N-(3-(4-(1-isoquinolylmethyl)phenyl)-2-propynyl)acetamide in the acylated GIP detection system. In WO 02/04626, which discloses the GWT1 gene, these two compounds are listed in Table 1, and described in Example B2 and Example B60 respectively.

Fig. 4 is a photograph showing the results of determining the GPI acylation inhibitory activities of N-(3-(4-(1-isoquinolylmethyl)phenyl)propyl)-N-methylacetamide and 5-butyl-2-(1-isoquinolylmethyl)phenol in the acylated GIP detection system. In WO 02/04626, which discloses the GWT1 gene, these two compounds are listed in Table 1, and described in Example B73 and Example B85 respectively.

#### Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using Examples, but it is not to be construed as being limited thereto.

##### 15 [Example 1] Preparation of membrane fraction expressing GWT1 protein

###### (1) Preparation of GWT1 expression plasmid

The vector for expressing in *S. cerevisiae*, YEpl352GAPII vector, was prepared by inserting a GAPDH promoter and a GAPDH terminator, both derived from pKT10 (Tanaka *et al.*, Mol. Cell Biol., 10: 4303-4313, 1990), into the multi-cloning site of YEpl352; and replacing the multi-cloning site with that of pUC18. Furthermore, to facilitate the insertion of the GWT1 gene, YEpl352GAPIIClaIΔSal vector was prepared by substituting the ClaI site for the SalI site in the multi-cloning site.

The *S. cerevisiae* GWT1 gene comprising the nucleotide sequence of SEQ ID NO: 1 was amplified using the primers of SEQ ID NOs: 15 and 16. The resulting PCR product was inserted into the multi-cloning site of YEpl352GAPIIClaIΔSal vector to prepare the GWT1 overexpression plasmid.

###### (2) Preparation of *S. cerevisiae* GWT1 gene-deficient strain Δgwt1

A his5 cassette comprising GWT1 sequences at both ends was amplified by PCR using the *S. pombe* his5 gene (Longtine MS *et al.*, Yeast, 14: 953-961, 1998) as a template and the sequences of SEQ ID NOs: 17 and 18 as primers.

*S. cerevisiae* cells were cultured and harvested, and then subjected to transformation with the PCR products described above. Then, the cells were cultured in SD(His-) medium at 30°C for five to seven days to obtain GWT1 gene-deficient strain Δgwt1.

###### 35 (3) Preparation of GWT1-expressing cells

Cells of the  $\Delta$ gwt1 strain were cultured while shaking in yeast extract-polypeptone-dextrose (YPD) medium at 30°C. The cells were harvested in the late logarithmic growth phase and then washed. The expression plasmid for GWT1 was introduced to the  $\Delta$ gwt1 strain cells by the lithium acetate method (YEAST MAKER™ Yeast Transformation System (Clontech)).  $\Delta$ gwt1 strain overexpressing the GWT1 gene was obtained by culturing the cells in SD(ura-) medium at 30°C for two days.

5 (4) Preparation of membrane fraction

Wild-type *S. cerevisiae* strain, the GWT1 gene-deficient strain  $\Delta$ gwt1, and the strain 10  $\Delta$ gwt1 into which the GWT1 overexpression plasmid was introduced were each cultured in 100 ml of YPD medium shaken at 24°C, and then harvested in the middle logarithmic growth phase (OD<sub>600</sub>= 1 ~ 3). The fungal cells were washed with TM buffer (50 mM Tris-HCl (pH 7.5) and 2 mM MgCl<sub>2</sub>), and then suspended in 2 ml of TM buffer + protease inhibitor (1 tablet of Complete™ (Roche) / 25 ml). 1.5 ml of glass beads was added to the suspension. The 15 mixture was vortexed for 30 seconds, and then placed on ice for 30 seconds. These procedures were repeated ten times to disrupt the fungal cells. The cell homogenate was transferred into a new tube, and centrifuged at 1000 g at 4°C for five minutes to precipitate the glass beads and undisrupted fungal cells. The supernatant was transferred to another tube, and centrifuged at 13 000g at 4°C for 20 minutes to precipitate the membrane fraction comprising organelles (total 20 membrane fraction). The resulting precipitate was used as the membrane fraction.

(5) Detection of acylated GPI

In the GPI biosynthesis reaction pathway, it is known that N-acetyl-glucosaminyl-phosphatidylinositol (GlcNAc-PI) is deacetylated to generate 25 glucosaminyl-phosphatidylinositol (GlcN-PI), to which an acyl group is then added to generate glucosaminyl-acylphosphatidylinositol (GlcN-(acyl)PI) (Fig. 1). The present inventors thus tested whether the Gwt1 protein was involved in this transacylation reaction using the method described below.

The membrane fraction preparation (300 µg protein) was diluted with a buffer 30 consisting of 50 mM Tris-HCl (pH7.5), 2 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 1 mM ATP, 1 mM Coenzyme A, 21 µg/ml tunicamycin, 10 µM nikkomycin Z, and 0.5 mM Dithiothreitol. The solution was adjusted to a total of 140 µl for use as a reaction solution. After incubating the solution at 24°C for 15 minutes, 15 µCi UDP-[<sup>14</sup>C]GlcNAc was added to the tube and then 35 incubated at 24°C for another one hour. 1 ml of chloroform:methanol (1:2) was added to the solution and stirred to stop the reaction. Then, lipid was extracted from the solution, dried, and desalted by butanol extraction. Acylated GPI (GlcN-(acyl)PI), non-acylated GPI (GlcN-PI),

and GPI which was neither acylated nor deacylated (GlcNAc-PI) were separated by thin-layer chromatography (HCl<sub>3</sub>/CH<sub>3</sub>OH/1 M NH<sub>4</sub>OH (10:10:3)). Each spot was detected by autoradiography.

As a result, as shown in Fig. 2, a spot for acylated GPI was not detected in the GWT1 gene-deficient strain ( $\Delta$ gwt1), while it was detected in the wild-type strain. The spot for acylated GPI was also detected in the GWT1 gene-introduced  $\Delta$ gwt1 strain, showing that this strain had recovered ability to acylate. These findings indicate that the Gwt1 protein is an enzyme that catalyzes transacylation to GPI.

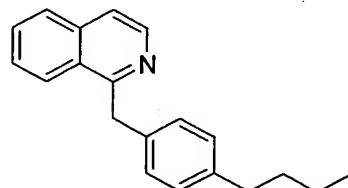
The above-described results suggest that the intensity of the spot for acylated GlcN-(acyl)PI is reduced or disappears when a compound having the activity of inhibiting the activity of GWT1 gene products is present in a system for assaying GPI synthase activity. Accordingly, compounds inhibiting the enzymatic activity of a GWT1 gene product, as well as compounds inhibiting the synthesis of fungal cell walls, can be screened using the intensity of GlcN-(acyl)PI spots as an indicator.

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#### (6) Screening for compounds that inhibit acylation

The compounds below are added to the acylated GIP detection system described in (5) to measure the activity of inhibiting GPI acylation. These compounds are described in Example B2, Example B60, Example B73, and Example B85 in WO 02/04626, which discloses the GWT1 gene. In WO 02/04626, these compounds are also listed in Table 1, which shows their inhibition activity in a reporter system reflecting the activity of the GWT1 gene products. The structures of these compounds are shown below:

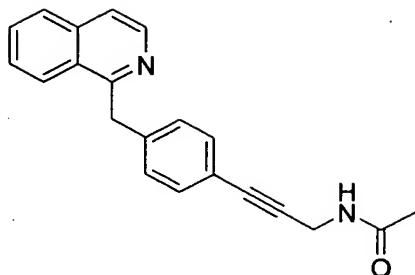
The compound described in Example B2: 1-(4-butylbenzyl)isoquinoline



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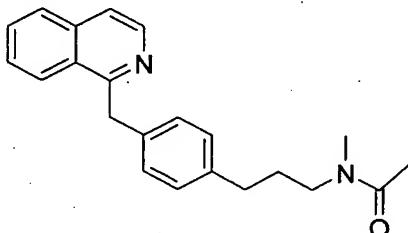
The compound described in Example B60:

N-(3-(4-(1-isoquinolylmethyl)phenyl)-2-propynyl)acetamide



The compound described in Example B73:

N-(3-(4-(1-isoquinolylmethyl)phenyl)propyl)-N-methylacetamide



5 The compound in Example B85: 5-butyl-2-(1-isoquinolylmethyl)phenol



The assay results are shown in Figs. 3 and 4. Of the compounds listed in Table 1 in WO 02/04626, the compounds described in Example B2 and Example B85, with inhibitory activities at IC50 of 1 µg/ml or less, showed a dose-dependent decrease in the spot intensity of acylated GPI. The spot intensity of the compound described in Example B73, with IC50 of 50 µg/ml, was not observed to decrease.

These results indicated that compounds inhibiting the enzymatic activity of GWT1 gene products can be screened by using the assay system for GPI acylation.

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#### Industrial Applicability

The present invention makes it possible to screen for compounds that inhibit the transport of GPI-anchored proteins to fungal cell walls by using a simple assay of transacylation activity.

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